

## Bacterial microcompartment-mediated ethanolamine metabolism in Escherichia coli urinary tract infection

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1 **Bacterial microcompartment-mediated ethanolamine metabolism in *E. coli* urinary**  
 2 **tract infection**

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23  
 24

25 **Abstract**

26 Urinary tract infections (UTIs) are common, in general caused by intestinal Uropathogenic *E.*  
27 *coli* (UPEC) ascending via the urethra. Microcompartment-mediated catabolism of  
28 ethanolamine, a host cell breakdown product, fuels competitive overgrowth of intestinal *E.*  
29 *coli*, both pathogenic enterohaemorrhagic *E. coli* and commensal strains. During UTI urease  
30 negative *E. coli* thrive, despite the comparative nutrient limitation in urine. The role of  
31 ethanolamine as a potential nutrient source during UTI is understudied. We evaluated the role  
32 of metabolism of ethanolamine as a potential nitrogen and carbon source for UPEC in the  
33 urinary tract. We analysed infected urine samples by culture, HPLC, qRT-PCR and genomic  
34 sequencing. Ethanolamine concentration in urine was comparable to the most abundant  
35 reported urinary amino acid D-serine. Transcription of the *eut* operon was detected in the  
36 majority of urine samples screened containing *E. coli*. All sequenced UPECs had conserved  
37 *eut* operons while metabolic genotypes previously associated with UTI (*dsdCXA*, *metE*) were  
38 mainly limited to phylogroup B2. *In vitro* ethanolamine was found to be utilised as a sole  
39 source of nitrogen by UPECs. Metabolism of ethanolamine in artificial urine medium (AUM)  
40 induced metabolosome formation and provided a growth advantage at the physiological  
41 levels found in urine. Interestingly, *eutE* (acetaldehyde dehydrogenase) was required for  
42 UPECs to utilise ethanolamine to gain a growth advantage in AUM, suggesting ethanolamine  
43 is also utilised as a carbon source. This data suggests urinary ethanolamine is a significant  
44 additional carbon and nitrogen source for infecting *E. coli*.

45

## 46 **Introduction**

47 Urinary tract infection is a common condition with an estimated 150 million episodes  
48 globally per annum (1). The most common identified cause is infection by uropathogenic  
49 *Escherichia coli* (UPEC) strains (2, 3). The currently accepted paradigm for uncomplicated  
50 urinary tract infection is that these *E. coli* strains residing in the gut as commensals  
51 successively colonise the perineum (4), the urethra and then the bladder, where the  
52 production of bacterial toxins and the host immune response lead to tissue damage and  
53 symptoms such as frequency and dysuria (2). Further ascending infection to colonise the  
54 kidney with more local tissue damage causing pyelonephritis and bacteraemia occurs in a  
55 small percentage of cases.

56

57 Common genetic features have been noted in a variety of *E. coli* strains causing infections  
58 outside the gastrointestinal tract, including UPEC, and these are collectively termed ExPEC  
59 (extraintestinal pathogenic isolates of *E. coli*) (5, 6). Panels of genes whose presence is  
60 associated with any *E. coli* infection outside the gastrointestinal tract (7), or specifically  
61 urinary tract infection (8), have been assembled by genetic comparison of *E. coli* strains  
62 isolated from the gut with those isolated from urine and other extraintestinal sites and those  
63 known to be virulent in different animal models. However, the mechanism by which these  
64 factors are involved in pathogenicity is obscure.

65

66 In the pathogenesis of *E. coli* urinary tract infection rapid invasion of bladder cells occurs  
67 with formation of intracellular bacterial communities (IBCs) with biofilm-like properties  
68 which initiate the infective process (9, 10). This bottleneck reduces diversity and has  
69 prevented global searches by signature tagged mutagenesis for key genetic factors required  
70 for infection (11). Assessing genome-sequenced clinical *E. coli* urinary isolates in a mouse

71 model of urinary tract infection showed that no set of genes was predictive of virulence in the  
72 model (12), including genes previously specifically associated with urovirulence.

73

74 Rapid growth has been shown to be characteristic of early phase *E. coli* infection in the  
75 urinary tract (13), suggesting securing nutrition in the urinary tract is a key part of *E. coli*  
76 pathogenesis. *E. coli* requirements for central carbon metabolism in the urinary tract have  
77 been explored by competition studies with selected mutants in murine models. Interruption of  
78 gluconeogenesis (*pckA*) or the TCA cycle (*sdhB*) reduces fitness of *E. coli* to infect (14). This  
79 is in contrast to the nutrient rich intestine, where glycolysis (*pgi*) or the Entner-Doudoroff  
80 (*edd*) pathway are required for colonisation fitness (15).

81

82 Some metabolic loci have been linked to UPEC pathogenesis. D-serine is an abundant amino  
83 acid in human urine, present at a mean concentration of 0.12 mM (16), and up to 1mM in  
84 some cases (17), much higher than intestinal content levels. Some *E. coli* strains can  
85 metabolise D-serine to pyruvate and ammonia (18), allowing it to be a sole carbon and  
86 nitrogen source in vitro (19). This is conferred by possession of a complete D-serine  
87 tolerance locus (*dsdCXA*) (20), where *dsdC* encodes a LysR-type transcriptional regulator  
88 (LTTR), *dsdX* a D-serine transporter (21) and *dsdA* a D-serine dehydratase. ExPEC strains  
89 usually encode a full *dsdCXA* locus, while enteric pathogenic *E. coli* frequently have a  
90 truncation after *dsdC* (22). In the absence or truncation of this locus, D-serine shows  
91 reversible toxicity for *E. coli* causing growth arrest at concentrations of 0.1 mM and above *in*  
92 *vitro* (23).

93 A metabolic regulatory polymorphism has been associated with cobalamin-independent  
94 methionine synthase (MetE) in UPEC. A promoter polymorphism (*sra* or short regulatory

95 allele) upstream of the *metE* gene in these strains is associated with increased *metE* induction  
96 and enhanced ability to grow in urine *in vitro* (24).

97

98 Mutational analysis of a subset of *E. coli* genes showing a marked (>fourfold) increase in  
99 transcription in infected patient urine compared to growth in urine or Luria Broth (LB) (25)  
100 showed that that their knockout caused a fitness defect in the urinary bladder in a mouse  
101 model of ascending urinary infection. The most marked defects were with knockout of the  
102 *cus* (copper resistance) and *eut* (ethanolamine uptake and metabolism) operons.

103

104 The *eut* operon is part of the conserved *E. coli* core genome (26) having arrived in  
105 Enterobacterales by horizontal transfer (27). It contains seventeen genes including the  
106 positive transcriptional regulator *eutR*. The operon encodes enzymes required for  
107 ethanolamine metabolism and includes structural shell protein genes for the synthesis of thin  
108 porous protein shells enclosing the enzymes as bacterial microcompartments  
109 (metabolosomes) in the cytoplasm (28–30) (Fig 1A). Experiments largely conducted with  
110 *Salmonella enterica* (which contains the same operon) suggest that the enzymic breakdown  
111 of ethanolamine to ammonia (a nitrogen source) and acetaldehyde occurs within the  
112 metabolosome, with the toxic effects and evaporative loss of acetaldehyde minimised by  
113 microcompartment enclosure and onward metabolism to ethanol and acetyl-CoA (a carbon  
114 source)(30, 31). Some acetyl-CoA is further metabolised to acetyl phosphate and acetate  
115 within the metabolosome, and some is available to enter central metabolism (32).

116 Ethanolamine in the gastrointestinal tract utilised by this pathway gives a competitive  
117 advantage to Enterohaemorrhagic *E. coli* (33) and *Salmonella enteritidis* (34). Recently it has  
118 been shown that *E. coli* ethanolamine metabolism is essential for bladder colonisation in a  
119 murine model of ascending UTI (35). The mechanism was suggested to involve resistance to

120 innate immunity because the colonisation advantage of wild type UPEC over a  $\Delta eutR$  mutant  
121 was abolished in neutrophil-depleted mice. Clearance of an isogenic  $\Delta eutR$  mutant *E. coli*  
122 from the bladder coincided with peaking myeloperoxidase levels. However, resistance to  
123 hydrogen peroxide was unchanged in the  $\Delta eutR$  mutant.

124

125 In this study we evaluated the role of microcompartment-mediated ethanolamine metabolism  
126 in clinically infected urine samples and in laboratory cultures of *E. coli* strains isolated from  
127 infected urine. The *eut* operon was induced in infected urine, and ethanolamine was present  
128 in urine at a level that enhanced *E. coli* growth *in vitro*. Metabolosomes were visible on  
129 TEM in a UPEC strain grown with ethanolamine. Inactivation of the *eut* operon reduced  
130 growth of a UPEC strain in ethanolamine-containing nitrogen-limited minimal medium and  
131 growth and competitiveness in ethanolamine-containing artificial urine medium. Selective  
132 mutation of individual *eut* genes suggested that ethanolamine provided a carbon source in this  
133 artificial urine medium. In summary, we have identified that microcompartment-mediated  
134 metabolism of ethanolamine present in urine can give *E. coli* a growth advantage by  
135 providing an additional carbon and nitrogen source.

136

## 137 **Methods**

138

### 139 **Bacterial strains and culture conditions**

140 Clinically infected urine samples received at Cork University Hospital (CUH) containing  
141 visible bacteria and white cells were selected and anonymised. The protocol was approved by  
142 the Clinical Research Ethics Committee of the Cork Teaching Hospitals ref ECM 4 (c)  
143 12/08/14. A further 12 specimens of macroscopically clear urine with no bacteria or white  
144 cells were selected as controls. Following initial culture on CLED agar pure colonies

145 subcultured on Columbia blood agar were identified by MALDI-TOF using a Microflex LT  
146 mass spectrometer (Bruker Daltonik) and the MALDI Biotyper software package (version  
147 3.0). Antimicrobial sensitivity was determined by the VITEK® 2.0 system (Biomérieux)  
148 using EUCAST breakpoints. Strains used for gene inactivation or competitive growth assays  
149 are listed in Table 1. Sixty-one *E. coli* strains were isolated, and whole genome sequences  
150 obtained for 47 strains.

151

152 *E. coli* were routinely cultured in LB broth at 30 °C or 37 °C with aeration. To determine the  
153 ability to utilise ethanolamine, strains were cultured at 37 °C in modified M9 minimal media  
154 (33) containing 10 mM ethanolamine hydrochloride and 200 nM cobalamin with the addition  
155 of either 20mM glycerol or 20 mM ammonium chloride. Automated growth count cultures  
156 were incubated in 96-well plates in triplicate and OD<sub>600</sub> measured using a Biotek Eon  
157 Microplate Spectrophotometer over 48 hours. Manual growth curves were measured in 35 ml  
158 volumes with spectrophotometric analysis of 1 ml aliquots.

159

160 **Competition experiments** were carried out in a published liquid artificial urine medium  
161 (AUM) (36) and with the same medium with added ethanolamine hydrochloride at 0.5 mM,  
162 and 10 mM, with cell counts on LB agar. Pre-cultured *E. coli* strains were incubated in LB  
163 with antibiotics where appropriate. The cultures were washed in PBS and resuspended in  
164 AUM. Approximately equal concentrations of the wild type and isogenic mutant were used to  
165 inoculate AUM with ethanolamine as indicated in the text to give an approximate starting  
166 OD<sub>600</sub> of 0.1. The co-cultures were incubated at 37°C with aeration and at each time point  
167 the co-culture were diluted 10-fold in PBS and plated on to LB agar. The dilutions were  
168 plated onto LB agar and onto LB agar containing kanamycin to determine the concentration  
169 of each strain of *E. coli*. The plates were incubated overnight at 37°C and the CFU calculated.



170 The wild type CFU was calculated by subtracting the number of CFU resistant to kanamycin  
171 from the number of CFU on LB agar plates. The experiment was repeated three times and a  
172 competitive index was calculated as follows:

173

$$CI = \frac{eut\ mutant\ CFU\ recovered / Wild\ Type\ CFU\ recovered}{eut\ mutant\ CFU\ inoculum / Wild\ type\ CFU\ inoculum}$$

174

175 A competitive index below 1 indicates that the wild type was outcompeting the mutant strain  
176 at that time point. The CI at time zero is by definition 1.0 . Growth of *eut* operon mutants was  
177 compared with wild-type strains in M9 minimal medium with 0.5 mM and 10 mM  
178 ethanolamine and AUM with 10 mM ethanolamine.

179

## 180 **Mutants**

181 To generate deletion mutants, BW25113 knockout *E. coli* strains for the genes of interest  
182 were obtained from the Keio collection (37). Mutations were transferred to UPEC strain U1  
183 by P1 vir phage transduction (38) In brief, lysogen strains were prepared by incubating P1  
184 lysate with the donor strain for 30 minutes at 30°C with 5µl of 1M CaCl<sub>2</sub> and the culture  
185 were plated on kanamycin selective agar. The resulting colonies were used to prepare the  
186 lysate for transduction. Lysogen colonies were grown overnight in 2ml of LB at 30°C. The  
187 precultures were used to inoculate LB and grown until reaching an OD<sub>600</sub> of 0.2. The cultures  
188 were incubated in 46°C for 20 minutes with shaking before being moved to 37°C until  
189 complete lysis. Bacteria were centrifuged out of the culture and the supernatant was stored  
190 with chloroform to prevent bacterial growth. Overnight cultures of the recipient strain were  
191 resuspended in transduction buffer (10mM MgSO<sub>4</sub>, 5mM CaCl<sub>2</sub>) and 100µl of cells were  
192 incubated with lysate and incubated at 37°C for 30 minutes. Sodium citrate was added  
193 following this incubation and for a further hour. The cells were washed in LB before being

194 plated onto LB agar plates. Strains were selected for kanamycin resistance and transductants  
195 were confirmed by genome sequencing and PCR using primers internal to the kanamycin  
196 gene and upstream and downstream of the disrupted gene (Supplementary Data Table S1).  
197 Complementation was with *E. coli* K-12 genes cloned in pCA24N from the ASKA library  
198 (39) induced by 0.01mM IPTG.

199

## 200 **Metabolic assays**

201 After culture, residual urine samples were separated into cell fraction and cell free  
202 supernatant by differential centrifugation and urine supernatants were filtered with a 0.2µm  
203 membrane to remove any remaining bacteria and stored at -80°C. Urine supernatants and  
204 culture supernatants were assayed for ethanolamine, acetate and ethanol by HPLC using an  
205 Agilent 1200 HPLC system with a refractive index detector. Urines collected from CUH and  
206 bacterial culture supernatants were filter sterilised at 0.2µm to remove bacteria before being  
207 stored at -80°C until the day of experimentation. Ethanolamine was measured by gradient  
208 HPLC after derivatisation with o-phthaldialdehyde (OPA) using a method adapted from  
209 Sturms et al. (40). The mobile phase consisted of Buffer A [10% methanol (Sigma-Aldrich) -  
210 90% 10mM Na<sub>3</sub>PO<sub>4</sub> (pH7.3) (Sigma-Aldrich)], and Buffer B [80% Methanol- 20% 10mM  
211 Na<sub>3</sub>PO<sub>4</sub> (pH7.3)]. Samples were prepared using an in-loop derivatization reaction where 6µl  
212 of sample were taken up followed by 6µl 10mg/ml OPA and 3-mercaptopropionic acid in  
213 0.4M boric acid (Agilent Technologies) and incubated at room temperature for 3 minutes.  
214 The samples were injected into a 4.6 by 100mm, 2.7µm pore Infinity Lab Poroshell HPH-  
215 C18 column (Agilent Technologies) and eluted with 5ml linear gradient from 50% Buffer B  
216 to 100% Buffer B followed by 5mls of 100% Buffer B at as constant flow rate of 1ml min<sup>-1</sup>.  
217 The excitation was detected at 224nm. A standard curve was created before each sequence

run. Identification of the peak and quantification was determined by comparison to retention time and standard curve.

Acetate and ethanol were measured by the same HPLC system. 10µl of sample was injected into a REZEX 8µm 8% H, Organic Acid Column (Phenomenex, USA) and eluted with 15ml of 0.01M H<sub>2</sub>SO<sub>4</sub> at a flow rate of 0.6ml min<sup>-1</sup>. The column was maintained at 65°C for the duration of the experiments. The identification of the substrate was determined by comparison of retention time to pure compound and concentrations were quantified by comparison to known standards.

#### **Transmission Electron Microscopy (TEM)**

This was carried out as previously described (41) After growth (as indicated in the text) bacteria cells were pelleted by centrifugation, to give a pellet no larger than 100µl in volume. The bacterial pellet was fixed in 2ml of 2.5% glutaraldehyde (Fluka) diluted in 0.1M Sodium cacodylate pH 6.8 (CAB) (bioWORLD). After incubation overnight at 4°C, bacteria were washed twice with 0.1 M CAB and suspended in fresh 2ml of 2.5% glutaraldehyde diluted in CAB. The bacteria were stained for 1 hour in 1 ml of 1% osmium tetroxide (w/v) (250µl 4% osmium tetroxide; 250 µl Milli-Q H<sub>2</sub>O; 500 µl 0.2 M CAB). The pellets were washed in 2ml Milli-Q H<sub>2</sub>O for 10 minutes twice before the pellets were dehydrated. Pellets were dehydrated through an ethanol (EtOH) gradient as follows: 50% EtOH (v/v) x 10mins; 70% EtOH x 10min; 90% EtOH x 10mins; 100% EtOH x 10mins three times and then the bacterial pellets were washed twice in propylene oxide for 10mins. The pellets were embedded into 1.5 ml propylene oxide: LV resin at 1:1 for 30 min followed by incubation 2 × 1.5 h in 100% freshly made agar LV resin. The pellets were resuspended in 1ml of 100% LV resin and transferred to a conical bottom tube. The bacterial pellet was centrifuged at 1100xg for 5mins and was left to incubate at 60°C for 24 hours. Bacteria were sectioned to

243 60-70 nm with a diamond knife on a LEICA-EM-UC7 ultramicrotome. Sections were  
244 collected on 400 mesh copper grids and stained with 4.5% (w/v) uranyl acetate in 1% acetic  
245 acid (v/v) for 45mins and Reynolds lead citrate for 7 mins at room temperature. Sections  
246 were then observed on a Jeol 1230 transmission electron microscope operated at an  
247 accelerating voltage of 80kV and imaged with a Gatan OneView digital camera.

248

#### 249 **DNA sequencing and sequence analysis, statistics**

250 DNA was extracted from overnight cultures in LB and extracted using Qiagen DNEasy  
251 Blood and Tissue (Qiagen) with RNase A treatment (Sigma). Bacterial genome sequencing  
252 was carried out by MicrobesNG (see acknowledgements) using Illumina HiSeq 2500  
253 2x250bp paired-end reads. Reference genomes were identified using Kraken (42) and reads  
254 mapped using BWA-MEM (43). De novo read assembly was achieved using SPAdes (44),  
255 with read mapping back to the resultant contigs, using BWA-MEM for quality metrics.  
256 Automated annotation was performed using Prokka (45). Sequencing data are available for  
257 download from the EBI European Nucleotide Archive under BioProject accessions  
258 PRJEB31941, PRJEB31942, PRJEB31943, and PRJEB31944.

259

260 Phylogenetic trees were generated from contig sequences with Parsnp (Harvest tool suite  
261 (46)) and edited with ITOL (47). Parsnp produces a core genome alignment and identifies  
262 SNPs for tree generation by FastTree2 (48) using SH-like (Shimodaira-Hasegawa) local  
263 supports for bootstrapping. Alignment with 32 reference genomes known to be representative  
264 of six *E. coli* phylogroups (49) was used for phylogroup assignment. Gene presence in  
265 genomes was taken as >75% identity in BLASTN search over the full reference gene  
266 sequence length. Binary matrices were prepared representing sequenced genomes with PUF  
267 gene presence scored as 1 and absence as zero, and phenotypic antimicrobial resistance

268 scored as 1 and sensitivity as zero. Two-dimensional cluster analysis on these matrices was  
269 performed with the R software package using complete linkage clustering on the Jaccard  
270 Distance. The resulting cladograms and heat maps were visualised with ITOL (47). All other  
271 statistical analyses presented were generated with GraphPad Prism 7.

272

### 273 **RNA and RT-PCR**

274 RNA was extracted from bacterial pellets using the Zymo Fungal/Bacterial Mini Prep kit and  
275 from Eukaryotic cells using the Quick-RNA MiniPrep kit, following the manufacturer's  
276 instructions. After extraction genomic DNA was digested using the TURBO DNA-free  
277 (Ambion) DNase 1 treatment. The RNA was quantified using a Nanodrop 1000  
278 spectrophotometer. cDNA was synthesised by reverse transcription carried out in nuclease  
279 free 96-well plates. RNA was diluted using molecular grade H<sub>2</sub>O (Sigma-Aldrich) to a final  
280 concentration from of 100ng  $\mu\text{l}^{-1}$  in a 10 $\mu\text{l}$  volume. The RNA was mixed with: cDNA  
281 reaction was set up 4 $\mu\text{l}$  5x Reverse transcription buffer (Roche); 3 $\mu\text{l}$  Random Hexamer  
282 Primer (Roche); 2 $\mu\text{l}$  20mM dNTPs mix; and 1 $\mu\text{l}$  Reverse transcriptase/RNase Inhibitor to  
283 give a total volume of 20 $\mu\text{l}$ . The reaction mixture was incubated in a thermocycler in the  
284 following condition: 10mins at 25 °C; 30mins 55 °C; 5mins at 85 °C; hold at 4 °C. The  
285 cDNA was then diluted to 100 $\mu\text{l}$  and stored at -20 °C until use.

286

287 The universal probe library (Roche, Indianapolis USA) was utilized to design primers for  
288 quantitative PCR. The primers used in this study are listed in Data Supplement Table S1  
289 Amplification reactions were a mix of: 3 $\mu\text{l}$  of cDNA; 7 $\mu\text{l}$  TaqMan Probe Master buffer  
290 (Roche); 1 $\mu\text{l}$  20mM primer mix (L+R primers); 0.1 $\mu\text{l}$  probe(Roche);and 0.9  $\mu\text{l}$  molecular  
291 grade H<sub>2</sub>O to a make a final volume of 10  $\mu\text{l}$ . When the probe was not available a SYBR  
292 Green master mix was used which included: 3  $\mu\text{l}$  cDNA, 5  $\mu\text{l}$  2xSYBR Green I Master buffer

(Roche); 1 µl 20mM primer mix(L+R primers) and 1 µl molecular grade H<sub>2</sub>O to a final volume of 10 µl. All reactions were performed using a 384 well plate on the LightCycler®480 System (Roche) with molecular grade water included as a negative control. Thermal cycling condition were as follows: 50°C for 2 mins, 95°C for 10 mins followed by 45 cycles for 95°C for 10s, 60°C for 45 s and 72°C for 60 s. Relative gene expression was calculated using the 2- $\Delta\Delta C_t$  (50). X-fold changes in mRNA of target gene was quantified relative to *gyrA*.

300

### 301 **ELISA**

302 Frozen urines were analysed using Meso Scale Discovery (MSD) V-PLEX proinflammatory  
303 panel I and Cytokine Panel II (MSD, Rockville, MD) enzyme-linked immunosorbent assays  
304 (ELISAs). Assays were performed according to the manufacturer's instructions and measured  
305 using MESO QuickPlex SQ120. Calibrators were run in duplicate with the urines and used to  
306 form a standard curve. The concentration of cytokines in the urine were extrapolated from the  
307 standard curve. Values which fell below the limits of detection were excluded from statistical  
308 analysis.

309

### 310 **Results**

#### 311 **Ethanolamine is present in urine and infecting *E. coli* strains show *eut* operon induction**

312 One hundred and three clinically infected urine samples were selected from which 61 *E. coli*  
313 strains were isolated, 47 of which were sequenced and used for *in vitro* metabolic analysis.  
314 The mean concentration of ethanolamine in 54 clinically infected urine samples was 0.55  
315 mM (mean  $\pm$ 0.076) and 0.66 mM (mean  $\pm$ 0.155) in 12 control urine samples which were not  
316 clinically infected (contained no white cells or bacteria on microscopy) (Figure 1B). The  
317 difference between infected and control urines was not significant. In 24 *E. coli* infected

318 urine samples from which RNA was extracted, transcription of *eut* operon genes was detected  
319 in the majority of cases for *eutB* (88%) *eutS* (68%) and *eutR* (63%). Expression of *eutB*  
320 significantly correlated with the ethanolamine concentration in urine (Fig. 1C). Because of  
321 anonymisation individual patient details are not available. Audit of diagnostic urine  
322 specimens in our laboratory shows that 75% come from general practice, 25 % from hospital  
323 sources, and 75% overall from women.

324

325 **Clinically infected urine samples show stimulation of the host innate immune response .**

326 Cytokines IL-8 and IL-1 $\beta$  were detected in 81% of clinically infected urine samples and  
327 significantly increased in infected urines compared to non-infected urine (IL-1 $\beta$  P=0.0048,  
328 and IL-8 P<0.001, see Fig. S1 Supplemental material). Mean IL-6 levels were higher in  
329 infected urine than in non-infected urine but the difference was not significant (Data  
330 Supplement Fig. S1).

331

332 **Uropathogenic *E. coli* strains utilise ethanolamine *in vitro* resulting in enhanced growth,**  
333 **formation of bacterial microcompartments, and production of acetate and ethanol**

334 Forty-five out of 47 (96%) *E. coli* strains isolated from urine showed increased overnight  
335 growth with 10 mM ethanolamine as the sole nitrogen source in M9 minimal medium (Data  
336 Supplement Fig. S2). No increased growth was detected with 10 mM ethanolamine as a sole  
337 carbon source in M9 for four strains shown to actively metabolise ethanolamine as a nitrogen  
338 source (Data Supplement Fig.S2, S3). For these selected strains (U1, U13, U17, U38) growth  
339 in M9 medium with ethanolamine containing glycerol as a carbon source commenced after  
340 10 hours (Fig. 2A) with ethanolamine consumption from around eight hours (Fig. 2C).  
341 Addition of 10 mM ethanolamine to artificial urine medium (AUM) also increased growth of  
342 these strains (Fig. 2B) with consumption of ethanolamine from around four hours incubation

343 onwards (Fig. 2D). Acetate and ethanol were produced by *E. coli* U1 growth in both M9 and  
344 AUM media when ethanolamine was added (Fig. 2C,D) and corresponded with induction of  
345 the *eut* operon at 4 and 8 hours of incubation with ethanolamine in AUM (Data Supplement  
346 Fig. S4). TEM of *E. coli* U1 grown in AUM with added ethanolamine showed 100-130 nm  
347 cytoplasmic inclusions with straight edges (Fig. 3A) in the majority of cells visualised (43/69  
348 = 62%). These structures are typical of bacterial microcompartments. They were not  
349 observed in cells grown in the absence of ethanolamine (Fig. 3B) and were seen in a minority  
350 of cells grown in minimal medium with ethanolamine (Supplementary data Fig. S5). The  
351 difference in TEM appearances between M9 and AUM medium may be growth phase-  
352 related. Cells were collected for TEM at 8 hours incubation which is approximately the  
353 starting time for ethanolamine consumption in M9 minimal medium, but the time of most  
354 rapid consumption in AUM (Fig. 2). Acetate was detected in nearly all infected urine samples  
355 tested (Supplementary Data Fig. S8).

356  
357 **The effect of inactivation of individual enzyme-encoding genes in the *eut* operon**  
358 **suggests ethanolamine growth stimulation in artificial urine medium is due to provision**  
359 **of an additional carbon source**

360  
361 Mutation of the *eut* operon genes *eutB* and *eutE* was achieved in strain U1 (Table 1). *eutB*  
362 encodes the heavy chain component of ethanolamine ammonia lyase required to liberate  
363 ammonia from ethanolamine, and *eutE* encodes a reversible acetaldehyde dehydrogenase,  
364 acting after *eutBC* in the ethanolamine catabolism pathway (see schematic, Fig. 1A). EutE is  
365 required to generate acetyl-CoA, which is the route for carbon assimilation from  
366 ethanolamine (Fig. 1A).



367 Growth stimulation in nitrogen-limited minimal (M9) medium by addition of ethanolamine  
368 (0.5 mM or 10 mM) was abolished by deletion of *eutB* in U1 and retained after deletion of  
369 *eutE* (Fig. 4A, 4B, Data Supplement Fig. S6, Table 2). RT-PCR showed that ethanolamine  
370 induced *eutE* transcription in the *eutB* mutant and vice versa, demonstrating that these were  
371 not polar mutations (Supplementary Data Fig. S4). Ammonia generation from ethanolamine  
372 alone is therefore sufficient to stimulate *E. coli* U1 growth in nitrogen-limited minimal (M9)  
373 medium. Complementation of the *eutB* mutant restored the wild-type phenotype in  
374 ethanolamine-containing minimal medium (Fig. 4A).

375 In contrast to this phenotype in nitrogen-limited minimal (M9) medium, in AUM medium  
376 which contains 25 mM ammonium chloride and no glycerol as carbon source, growth  
377 stimulation by ethanolamine was absent in U1  $\Delta$ *eutE*, although ethanolamine was still  
378 metabolised by this strain (Fig. 4D,E, Table 2). Growth enhancement by ethanolamine in  
379 AUM was restored by *eutE* complementation. Therefore, in AUM, unlike nitrogen limited  
380 M9, the growth stimulation conferred by ethanolamine metabolism is not due to ammonia  
381 generation, but appears to be caused by the provision of an additional carbon source from  
382 acetyl-CoA.

383 **A functional *eut* operon is essential for competitive growth of a UPEC strain in the**  
384 **presence of ethanolamine *in vitro***

385 Competitive growth assays in AUM containing 10 mM ethanolamine between wild type *E.*  
386 *coli* strain U1 and  $\Delta$ *eutB* and  $\Delta$ *eutE* mutants showed a significant advantage for the wild-type  
387 after 32 hours (incorporating a 24-hour subculture) for both mutants (Fig. 5). The  $\Delta$ *eutE*  
388 mutant showed a significant disadvantage from 12 hours onwards. The competitive index  
389 (CI) of both mutants at all time intervals from 12 hours onwards was less than 0.8 (Data  
390 Supplement Table S2) No significant difference was found in competitive growth between

391 wild type and mutants in AUM with 0.5 mM ethanolamine (Data Supplement Fig. S7) or in  
392 the absence of ethanolamine (data not shown).

393

394 **The *eut* operon is conserved in all UPEC strains sequenced while putative urovirulence**  
395 **factors and metabolic polymorphisms previously associated with UPEC are**  
396 **phylogroup-related**

397 A SNP-based tree from a core genome alignment of the 47 urine *E. coli* isolates and 32  
398 representative reference strains by Parsnp (46) assigned all urine strains to phylogroups (Fig.  
399 6). The largest single grouping of urine *E. coli* isolates was formed by 22 phylogroup B2  
400 strains (46%) (Fig.6), followed by 11 phylogroup D2 (23%), 7 A 15%), 4 B1(9%), 2 D1(4%)  
401 and one phylogroup E (2%). The tree shown used U7 from this study as the reference strain  
402 for SNPs and the core 79-genome alignment (47 from this study plus 32 phylogroup  
403 representatives) included 53% of the U7 genome. The same phylogroup assignments were  
404 found in trees generated with finished closed GenBank genome sequence strains from each  
405 phylogroup as the SNP reference strain, as expected (46).

406

407 The presence of a set of 31 previously described (12) putative virulence factors (PUFs)  
408 determined by BLASTN searching was used to score each of the 47 *E. coli* genomes. These  
409 represented a compilation of genes previously found to be enriched in UTI *E. coli* strains  
410 compared to other *E. coli* (51–54). All 31 PUFs were found in the set of genomes and the  
411 median PUF count was 13 (range 2-25). Phylogroup B2 *E. coli* urine isolates had higher PUF  
412 counts than non-B2 strains ( $P < 0.001$ , Mann-Whitney U test) (Figure 7A). Hierarchical  
413 clustering of PUF carriage profiles showed PUF profile patterns related to B2 clade  
414 membership, while clustering of antimicrobial resistance phenotypic profiles showed no  
415 obvious phylogenetic relationship (Figure 7B).

416

417 Regarding metabolic features, the *eut* operon was conserved in all 47 strains (Fig. 6B).

418 However, strain U71 contained a novel prophage in the same site as the CPZ-55 prophage

419 insertion between *eutA* and *eutB* characteristic of *E. coli* MG1655 (55) and other K-12420 lineage strains (Fig. 1A). Genome sequencing of the knock-out strains U1  $\Delta$ *eutB* and  $\Delta$ *eutE*,

421 above, revealed the expected single gene deletions (marked by a kanamycin cassette).

422

423 A short *metE* regulatory allele was present in 30 strains and a complete D-serine tolerance424 locus (*dsdCXA*) was present in 29 strains (Fig. 6B). All strains contained a complete425 *yhaOMKJ* D-serine sensory locus. B2 strains were more likely to possess a short *metE*426 regulatory allele and a complete *dsdCXA* locus than non-B2 strains (2-sided P <0.0001 and

427 0.0022, respectively, Fisher's exact test).

428

429

430 **Discussion**431 The *E. coli* strains isolated from urine in this study were phylogenetically similar to

432 previously published urinary tract infection series, in that B2 and D2 were the commonest

433 two phylogroups (56). We report a lower proportion of B2 strains (46%) (Fig. 6) than

434 urosepsis and urinary tract infection studies from the USA and Spain (67%-69%) (12, 54, 57,

435 58), a similar proportion to Slovenia (50%) (59), and more than Denmark (34%) (60) and

436 China (19%) (56). The PUF profile association demonstrated with phylogroup B2 (Fig. 7) is

437 consistent with previous findings from a set of urinary tract infection isolates from the

438 USA (12). This study found that B2 strains not associated with urinary tract infection are also

439 enriched for these genes and that PUF profile does not correlate with virulence in animal

440 models of UTI (12). Phylogroup B2 strains are more likely than other phylogroups to

441 colonise the gut (61, 62) and these putative urovirulence factors may in fact be more  
442 important in the gut. Similarly, we found that the metabolic loci proposed to be helpful for  
443 growth in urine such as D-serine tolerance and short *metE* allele were also associated with  
444 phylogroup B2 (Fig. 6).

445

446 In contrast the *eut* operon was conserved in all isolates (Fig.6) and the ability to utilise  
447 ethanolamine *in vitro* was observed in 96% of strains (Supplementary Data Fig. S2). This is  
448 not surprising, because the *E. coli* core genome includes the *eut* operon (26). Therefore, the  
449 presence of ethanolamine accessible in urine is potentially a significant nutritional resource  
450 for all phylogroups of UPEC.

451

452 We found similar concentrations of ethanolamine in infected urine from patients 0.55 mM  
453 (mean  $\pm 0.076$ ) and non-infected urine controls 0.66 mM (mean  $\pm 0.155$ ) (Fig. 1B). The levels  
454 are consistent with previous reports on smaller numbers of samples from healthy controls  
455 using different methodology such as NMR (0.38 mM) (63) and LC/MS (0.47 mM) (64). The  
456 NMR study found ethanolamine in all 22 urine specimens processed (63). The lack of  
457 ethanolamine in a minority of our infected specimens (9/54, Fig.1B) may reflect limitations  
458 of the HPLC assay. The maximal ethanolamine concentration in bovine intestinal content  
459 (BIC, the filtered contents of jejunum and ileum), where enterohaemorrhagic *E. coli* has been  
460 shown to gain an *in vitro* competitive advantage by ethanolamine utilisation, is 2.2 mM (33).  
461 For comparison, D-serine is regarded as an abundant substrate for *E. coli* metabolism in  
462 human urine (65) where it has been reported at a mean concentration of 0.12 mM out of a  
463 total mean urine serine concentration of 0.33 mM (16).

464

465 We found evidence that ethanolamine in infected urine was sensed by *E. coli* with induction  
466 of the *eut* operon regulator *eutR*, and was being metabolised, with induction of the  
467 ethanolamine deaminase component *eutB* correlating with measured ethanolamine levels in  
468 urine (Fig. 1C,D). *In vitro*, UPEC strains produced acetate and ethanol when metabolising  
469 ethanolamine in both minimal medium and artificial urine medium (Fig. 2), as expected (Fig.  
470 1A) (66). Acetate was also detected in infected urine (Data Supplement Fig S8), as  
471 previously reported for infected urine samples with a variety of different bacterial causes  
472 (67). Acetogenic growth of *E. coli in vivo* is hypothesized to be an essential property in  
473 urinary tract infection (68, 69) and has been ascribed to metabolism of D-serine via pyruvate  
474 to acetyl-CoA and acetyl phosphate (68, 70). We propose the consistent presence of host-  
475 derived ethanolamine in urine at higher concentrations than D-serine also contributes to this  
476 phenotype. Acetate is an important regulator of *E. coli* gene expression (70) and the host  
477 immune response (71) and may contribute to the previously reported (35) phenotype linking  
478 the *eut* operon to resistance to innate immunity.

479  
480 TEM revealed that cells metabolising *E. coli in vitro* in AUM produced numerous plane-  
481 edged cytoplasmic inclusions typical of bacterial microcompartments (Fig. 3) in the majority  
482 of cells imaged. Although Eut microcompartments have been extensively imaged from  
483 *Salmonella enteritidis*, we are not aware of previous publications showing these from  
484 uropathogenic *E. coli*.

485  
486 Ethanolamine is not synthesized by mammals (72) and is obtained from the diet, with the  
487 ultimate source being plant and animal cell membranes. It is incorporated in  
488 phosphatidylethanolamine (PE), an aminophospholipid that is an essential constituent of cell  
489 membranes, particularly those of mitochondria and the endoplasmic reticulum (72). The

490 source for ethanolamine detected in urine has not been established. Cell lines *in vitro* release  
491 ethanolamine into culture medium from cell membrane turnover (73). Within the  
492 gastrointestinal tract available ethanolamine is assumed to derive from the breakdown of  
493 phospholipid from the turnover of the epithelium and dietary phospholipid (74). There is a  
494 constant supply of ethanolamine in urine in both health and infection (Fig. 1B) (63, 64), and  
495 the source in health seems unlikely to be cell turnover in the urinary tract, because this occurs  
496 at a relatively slow rate compared to the gastrointestinal tract. The cell membranes of  
497 neutrophils and bladder epithelial cells are additional potential sources in infected urine.

498

499 There is some evidence to regard *E. coli* as relatively nitrogen limited in the urinary tract  
500 because it lacks urease to metabolise the most abundant nitrogen source in urine. Induction of  
501 the high-ammonium affinity glutamine synthase and glutamate oxo-glutarate  
502 aminotransferase pathway (GS/GOCAT) for nitrogen assimilation occurs in *E. coli* infected  
503 urine (69, 75).

504

505 Because ethanolamine metabolism yields ammonia and acetate (Fig. 1A), in theory it should  
506 promote *E. coli* growth as either a sole carbon or nitrogen source. *E. coli* utilisation of  
507 ethanolamine as a sole nitrogen source in minimal media has been reported at concentrations  
508 of 30 mM (33). We found that 96 % of clinical UPEC strains showed utilisation of 10 mM  
509 ethanolamine as a sole nitrogen source (Fig. 2A, Data Supplement Fig.S3A). Contradicting  
510 the assertion that concentrations of ethanolamine below 1 mM (76) do not support growth of  
511 *E. coli*, we found that 0.5 mM, the level present in urine, could sustain small amounts of *E.*  
512 *coli* growth in nitrogen limited media (Supplementary Data Fig. S2, S6A). Utilisation of  
513 ethanolamine by *E. coli* strains as a sole carbon source *in vitro* is reported to require a high  
514 ethanolamine concentration (1 g l<sup>-1</sup>, 82 mM) (77). Even at this concentration some strains

515 showing active ethanolamine metabolism, for example the O157:H7 EHEC strain EDL933,  
516 have been reported as unable to use ethanolamine as a sole carbon source (33). Likewise, we  
517 found no *in vitro* growth promotion of known-ethanolamine metabolising UPEC strains by  
518 10 mM ethanolamine in carbon limited minimal media (Data Supplement Fig S3B).  
519 However, in artificial urine medium (AUM) where the nitrogen sources are urea and  
520 ammonia, and the carbon sources are amino acids, lactate and citrate (36), ethanolamine at 10  
521 mM and 0.5 mM (Fig 2B, 4B, 4C, Data Supplement Fig. S6B) promoted additional growth of  
522 *E. coli*.

523

524 In M9 nitrogen limited media the phenotype of *eutE* mutants showed that the ammonia  
525 liberated by the first reaction in ethanolamine metabolism catalysed by *eutBC* (Fig.1A) was  
526 sufficient for growth (Fig. 4B, Supplementary Data Fig. S6A). However, this was not  
527 sufficient for growth stimulation by ethanolamine in AUM where *eutE* was also required  
528 (Fig. 4D), suggesting generation of acetyl-CoA as an additional carbon source was  
529 responsible for additional growth in this medium. A second pathway for ethanolamine  
530 conversion to acetyl CoA has been predicted (but not defined) in *Salmonella enterica* from  
531 the ability of *eutBC* mutants to grow on ethanolamine as a carbon source in the presence of  
532 concentrations of carbon dioxide sufficient to change intracellular pH (31), but no carbon  
533 dioxide was provided in our experiments.

534

535 The observation that ethanolamine at 10 mM confers a competitive growth advantage on a  
536 wild type UPEC strain co-cultured with  $\Delta eutE$  and  $\Delta eutB$  mutants in artificial urine media  
537 (Fig. 5) also supports a role for acetyl-CoA generation in growth enhancement, because  
538 extracellular acetate or ammonia deriving from wild-type cells metabolising ethanolamine is  
539 apparently insufficient to confer growth enhancement on mutants in this medium. In contrast,

540 *E. coli* strains engineered for enhanced takeup of amino acids to grow faster on amino acids  
541 than a wild type strain when cultured in isolation, lose any growth advantage in co-culture  
542 with the wild type (78). This is because of extracellular ammonia leak from enhanced amino  
543 acid metabolism in the engineered strains providing nitrogen to the wild-type strain (78).

544

545 Although we did not demonstrate a competitive advantage of wild-type *E. coli* over *eut*  
546 operon mutants in co-culture in a physiological ethanolamine concentration of 0.5 mM (Data  
547 Supplement Fig. S7), this may well be due to methodological limitations. Following a 4 hour  
548 lag period, ethanolamine is removed from AUM medium by *E. coli* at a rate of approximately  
549 0.75 mM per hour (Fig. 2D), so any selective advantage due to 0.5 mM ethanolamine must be  
550 necessarily brief and difficult to detect in a competition assay based on batch culture.

551 However, *in vivo*, host-derived ethanolamine would be continuously passing into urine at the  
552 same time as bacterial ethanolamine catabolism. The level of ethanolamine seen in non-  
553 infected urine is maintained in infected urine (Fig. 1B) containing large numbers of *E. coli*  
554 with induced *eut* operons (Fig. 1C,D), suggesting it is an equilibrium level. The assertion that  
555 concentrations of ethanolamine below 1 mM (76), the level present in urine, do not support  
556 growth of *E. coli* is contradicted by our *in vitro* data in both minimal medium where  
557 ethanolamine functions as the sole nitrogen source (Fig. S6A) and the complex AUM where  
558 it appears to function as a carbon source additional to amino acids (Fig 6B). Ethanolamine in  
559 urine is an important nutritional resource that infecting uropathogenic *E. coli* can access to  
560 augment growth by microcompartment-mediated metabolism. These conserved metabolic  
561 pathways and structures distinct from the host offer opportunities for detection and treatment  
562 of infection.

563

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574

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578

579 **Tables**

580

581 **Table 1 Plasmids and strains in this study**

	Genotype/designation	Source
<b>Plasmids</b>		
pCA24N	High copy number expression vector, <i>cat</i>	NBRP <i>E.coli</i> , Japan (39)
pCA24N:: <i>eutB</i>	ASKA clone JW2434	“
pCA24N:: <i>eutE</i>	ASKA clone JW2439	“
<b>Strains</b>		
<i>E. coli</i> U1	<i>E.coli</i> Phylogroup A urine isolate	This study
<i>E.coli</i> JW2434-1	BW25113Δ <i>eutB</i>	Keio collection, Japan (37)
<i>E.coli</i> JW2439-1	BW25113Δ <i>eutE</i>	“
<i>E. coli</i> U1Δ <i>eutB</i>	Δ <i>eutB</i> :: <i>kan</i>	This study
<i>E. coli</i> U1Δ <i>eutE</i>	Δ <i>eutE</i> :: <i>kan</i>	This study
U2-U79 (46 strains)	<i>E. coli</i> urine isolates.	This study

582

583

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586

587

588 **Table 2** *In vitro* growth phenotype of wild type U1 and *eut* operon mutants with  
589 additional ethanolamine (Eth)

590

Genotype	M9 10mM Eth	M9 0.5mM Eth	AUM 10mM Eth	AUM 0.5mM Eth
U1 wild type	+	+	+	+
U1 $\Delta$ <i>eutB</i>	—	—	—	—
U1 $\Delta$ <i>eutE</i>	+	+	—	—
U1 $\Delta$ <i>eutB</i> / pCA24N:: <i>eutB</i>	+	+	+	ND
U1 $\Delta$ <i>eutE</i> / pCA24N:: <i>eutE</i>	+	+	+	ND

591

592

593 + growth enhancement compared to growth without ethanolamine. — no growth  
594 enhancement compared to growth without ethanolamine. \*Growth enhancement only  
595 observed after 30 hours. ND no data. M9 minimal medium, AUM Artificial Urine Medium.

596

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834 **Figure Legends Dadswell et al**

835

836 **Figure 1. Ethanolamine is present in urine and urinary ethanolamine concentration**  
837 **correlates with expression of *eut* operon genes in *E. coli* infected urine**

838 A. Microcompartment-mediated ethanolamine metabolic pathway and *eut* operon: black  
839 arrows metabolite translocation or reaction, dotted arrows metabolite translocation impeded  
840 by microcompartment, blue hexagon microcompartment, microcompartment associated  
841 enzymes in blue, cytoplasmic enzymes in black. Yellow arrows below show the *eut* operon  
842 (red arrow at prophage insertion hot spot). B. Ethanolamine concentration in urine. There is  
843 no significant difference in ethanolamine concentration between clinically infected urine  
844 samples and control non-infected samples (Mann-Whitney U-test). C. Correlation between  
845 ethanolamine concentration in infected urine and expression of *eutB* (relative to *gyrA*),  
846 Spearman's rank correlation coefficient  $r=0.815$ , \*\*\* $p < 0.001$ . D. Correlation between  
847 ethanolamine concentration in infected urine and expression of *eutR* (relative to *gyrA*),  
848 Spearman's rank correlation coefficient  $r=0.423$ .

849

850 **Figure 2. Ethanolamine metabolism promotes UPEC growth in nitrogen-limited**  
851 **minimal medium and Artificial Urine Medium (AUM).**

852 Aerobic growth of selected UPECs at 37 °C in: (A) ammonia-free modified M9 media with  
853 glycerol (20mM) (B) AUM. Hollow data points are without ethanolamine, solid data points  
854 with additional 10mM Ethanolamine. Concentration of ethanolamine (Eth) (green), acetate  
855 (red) and ethanol (blue) over time during U1 growth in (C) ammonia-free M9 media with  
856 glycerol (20mM) and (D) AUM, both supplemented with an initial 10mM ethanolamine.  
857 Values are Mean  $\pm$  SEM.  $N \geq 3$ .

858

859 **Figure 3. Growth of UPEC strain U1 in Artificial Urine Medium with ethanolamine**  
860 **promotes formation of bacterial microcompartments.**

861 Transmission electron microscopy following culture for eight hours. A. in AUM with 10 mM  
862 ethanolamine. B. In AUM alone. White arrows indicate microcompartments.

863

864 **Figure 4. *eutE* inactivation in UPEC strain U1 abolishes ethanolamine growth**  
865 **stimulation in AUM medium despite preserved ethanolamine catabolism**

866 A. Growth of U1, U1  $\Delta$ *eutB* mutant and complement in modified M9 plus 10 mM  
867 ethanolamine. B. Growth of U1, U1 $\Delta$ *eutE* mutant and complement in modified M9 with 10  
868 mM ethanolamine. C. Growth of U1, U1  $\Delta$ *eutB* mutant and complement in AUM plus 10  
869 mM ethanolamine. D. Growth of U1, U1 $\Delta$ *eutE* and complement in AUM with 10 mM  
870 ethanolamine. In A-D growth of U1 in control medium without ethanolamine is shown as  
871 open circles. E. Percentage change in ethanolamine concentration measured by HPLC over  
872 24 hours of U1, U1 $\Delta$ *eutB* and U1 $\Delta$ *eutE*, and their complements in AUM with initial 10mM  
873 ethanolamine. Significant difference with wild-type\*\*\*  $P < 0.001$ , 1-way ANOVA. All  
874 Values are Mean  $\pm$  SEM. N=3

875

876 **Figure 5. Inactivation of *eut* operon genes reduces competitiveness of *E. coli* UPEC**  
877 **strain U1 in artificial urine medium containing 10 mM ethanolamine**

878 Competition of U1 vs A: U1 $\Delta$ *eutB* with 10mM ethanolamine, B: U1 $\Delta$ *eutE* with 10mM  
879 ethanolamine. Mann-Whitney U test. \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$ . Values are Mean  $\pm$   
880 SEM. N=3.

881

882 **Figure 6. Phylogenetic distribution of *E. coli* urine isolates from this study and**  
883 **conservation of metabolic operons**

884 A. The phylogeny of 47 strains (taxon labels in red) isolated from infected urine analysed by  
885 core genome alignment using Parsnp with 32 reference strains representative of six *E.coli*  
886 phylogroups (taxon labels in black). Bootstrap values for all internal nodes were 1.0 apart  
887 from the node (0.25) between the reference strains APECO1 and IHE3034, which constitute  
888 the least diverged core genome pair in the reference set. Clade assignments shown in the  
889 vertical bar on the right. B. Parsnp alignment of the 47 strains alone, B2 phylogroup coloured  
890 blue. Vertical bars/circles indicate presence of a complete *eut* operon (red), a complete  
891 *dsdCXA* locus (green), and a short regulatory *metE* allele (grey) in each strain

892

893 **Figure 7 Carriage of PUFs (putative virulence factors) but not antimicrobial resistance**  
894 **is associated with clade B2 *E. coli* urine isolates**

895 A. PUF scores differ between B2 and non-B2 groups. Mann-Whitney U test \*\*\*  $p < 0.0001$   
896 B. Antimicrobial resistance scores (number of different antimicrobials to which the strain is  
897 resistant) does not differ between B2 and non-B2 groups  
898 C. Genome sequences of clinical urine *E.coli* isolates were screened for the presence of 31  
899 previously-described PUFs (y-axis labels) using BLASTN. Presence (black squares) or  
900 absence (grey squares) is shown for each PUF in relation to each isolate. Two dimensional  
901 hierarchical clustering shows PUF co-occurrence by strain (upper y-axis dendrogram) and  
902 PUF association with phylogeny (x-axis dendrogram). Clade B2 strains are indicated by  
903 white names on a black background (x-axis labels). Lower diagram shows hierarchical  
904 clustering of resistance (dark grey squares) and sensitivity (pale grey squares) to nine  
905 different antimicrobials (lower y-axis dendrogram) by strain phylogeny. Abbreviations as  
906 follows: Gent, Gentamicin; Nitro, Nitrofurantoin; Cipro, Ciprofloxacin; Levo, Levofloxacin;  
907 Tetra, Tetracycline; Cotrim, Cotrimoxazole; PipTaz, Piperacillin/tazobactam; Amp,  
908 Ampicillin; Coamox, Amoxicillin/clavulanic acid.



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